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**14. ABSTRACT**
Cells die in response to chemotherapeutic agents by activating a mitochondrial cell death pathway. This pathway leads to activation of proteases known as caspases through the release of cytochrome c from mitochodria. Once released, cytochrome c engages a cytoplasmic receptor known as Apaf-1, which oligomerizes and activates caspase 9. We demonstrated that breast cancer cells have apoptosomes which are hypersensitive to cytochrome c. Thus we have proposed to develop cytoplasmic variants of cytochrome c and/or small molecule cytochrome c mimetics to activate the apoptosome in breast cancer cells.

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Introduction

In response to chemotherapeutics, breast cancer cells initiate a cell death program culminating in the release of cytochrome c from mitochondria, binding of cytochrome c to a protein known as Apaf-1, and consequent Apaf-1-induced activation of a cell death protease known as caspase 9. We had reported previously that breast cancer cells were more sensitive to cytochrome c-induced apoptosis than their normal counterparts. Based on these findings, we wished to develop cytochrome c mimetic therapeutics for the treatment of chemoresistant breast tumors. We proposed both to modify cytochrome c itself as a potential therapeutic and screen for small molecules that might mimic cytochrome c.

Our original statement of work contained the following aims:

Task 1: Construction of cytochrome c and heme lyase variants allowing the production of cytoplasmically active cytochrome c (months 1-6)

Task 2: Testing of cytoplasmic cytochrome c/heme lyase in tissue culture and mice (months 12-20)

Task 3: Identification and testing of small molecule post-mitochondrial caspase activators (months 21-36)

As detailed in last year's report, for Task 3, we developed a novel single cell screen for regulators of caspase activity using the near-infrared caspase activity indicator (the caspase-3 IRDye, available from LI-COR Biosciences Inc) and the Xenopus oocyte system. Injection of these large cells is technically simple and thousands of cells can be injected in 4-5 hours. During this past year, we have thoroughly characterized this system and feel that it offers a robust assay for screening for small molecule cytochrome c mimetics, as proposed. As shown in Fig. 1, oocytes injected with this indicator and cytochrome c very rapidly cleave the substrate to produce an infrared signal detected by the LI-COR Odyssey reader. This signal is quite robust and enduring; caspase activation could be detected as little as 3 minutes after injection and signal persisted for 24 hours or more (Fig. 2). Similar results are obtained when we injected the pro-apoptotic Bcl-2 family member, t-Bid. Moreover, the signal was inhibited by co-injection of oocytes with the caspase inhibitor zVAD-fmk (Fig 3). We have acquired a library of bioactive compounds and have begun testing these (in pools) by manual injection to determine if we can identify pools that either rapidly induce apoptosis or that inhibit cytochrome c-induced

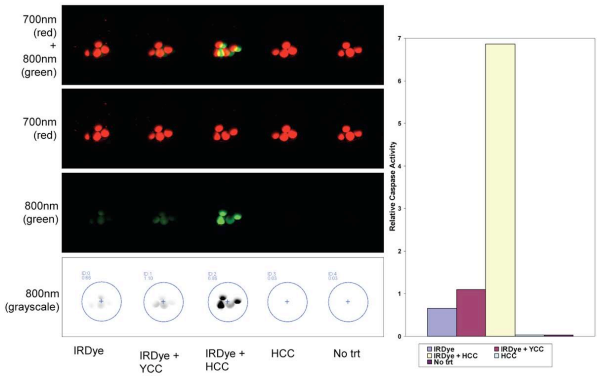


**Figure 1. Fluorescence in oocytes co-injected with the IRDye® and cytochrome *c*.** Oocytes were injected with either IRDye, IRDye and yeast cytochrome *c* (YCC), IRDye and horse cytochrome *c* (HCC), HCC alone, or nothing (No trt). Oocytes were imaged using the LI-COR Odyssey® thirty minutes after injection. Resulting images are shown on the left, demonstrating oocyte autofluorescence in the red channel (700nm) and IRDye signal in the green channel (800nm). 800nm signal from each group of oocytes was quantitated in the grayscale image (blue circles), and a graph from the obtained values is displayed on the right. As shown, significant signal is obtained only oocytes co-injected with IRDye and HCC.

apoptosis. Pools would then be deconvoluted by breaking down into smaller pools, etc.

We were not happy with the quality of the automated screening in cell free lysates that we were performing in house with a Duke collaborator. However, given all of our experience gained during the course of this funding, we have been able to miniaturize and optimize our assay sufficiently that we plan to leverage the knowledge gained from our DOD funding and apply to conduct a small molecule screen at an external site.

As described last year for **Task 1**, we produced cytochrome c and heme lyase variants that are cytoplasmic. Initially, we sought to do this systematically through the identification of residues important for mitochondrial import of these proteins. However, careful reading of the literature suggested that mutations disrupting mitochondrial import might also disrupt protein function. Therefore, we took a distinct tack, making fusion proteins that would alter either the N- or C- termini of the proteins, hoping that these might impede import, while allowing retention of function. Towards this end, we utilized a EGFP-cytochrome c, a cytochrome c-EGFP, an mCherry-heme lyase. These fusions offered the added advantage that we could visualize these proteins through fluorescence microscopy and/or sort cells expressing these proteins by fluorescence activated cell sorting. Interestingly, it had been reported previously that GFP-cytochrome c and cytochrome c-GFP could localize to mitochondria. However, we found that this was true in only a minor subset of cells expressing this construct. Rather, the majority was cytoplasmic (Fig. 4). As detected by fluorescence microscopy, the mCherry-heme lyase construct was entirely cytoplasmic. (See Fig 5). After completing Task1, we embarked on **Task 2**, expressing the cytoplasmic variants of cytochrome c and heme lyase in a variety of cell types to determine if this induces apoptosis. Although as reported last year, our preliminary

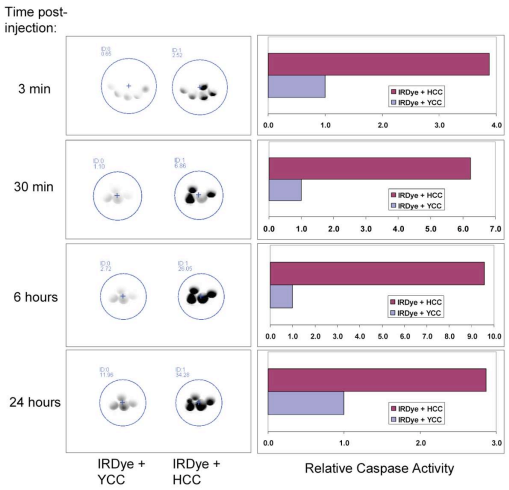


**Figure 2. Oocyte fluorescence can be detected from three minutes to twenty-four hours after injection.** Oocytes were microinjected with either IRDye + YCC or IRDye + HCC. Images of the injected oocytes were obtained on the LI-COR Odyssey® over time. Quantitated grayscale images of the 800nm signal are shown on the left for several time points, with corresponding graphs displayed on the right. Significant 800nm signal is obtained from as early as three minutes post-injection and is maintained for at least twenty-four hours selectively in the HCC-injected oocytes.

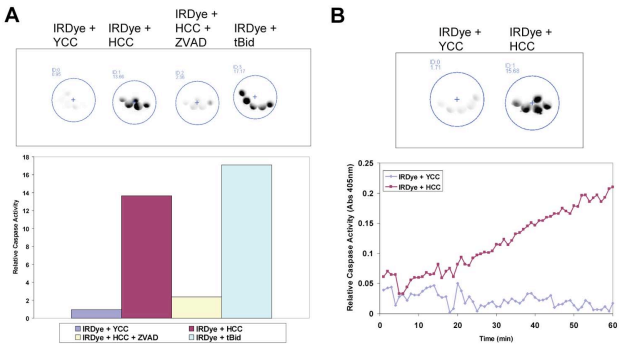


**Figure 3. Fluorescence in IRDye-injected oocytes is due to activation of effector caspases. A**, Oocytes were injected with either IRDye + YCC, IRDye + HCC, IRDye + HCC + the general caspase inhibitor ZVAD-FMK (ZVAD), or IRDye + a truncated form of Bid (tBid). A quantitated grayscale image of the 800nm signal and the corresponding graph are displayed. **B**, Oocytes injected as indicated (top) were flash-frozen thirty minutes following injection, lysates were prepared and caspase activation was measured using a colorimetric substrate (bottom).

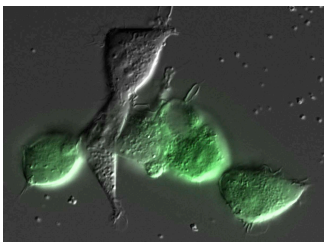

**Figure 4.** Cytosolic expression of cytochrome c fused to EGFP. HeLa cells were transfected with a construct encoding cytochrome c-EGFP and then visualized twenty-four hours later using fluorescence microscopy. Green fluorescence is detected throughout the cytosol in transfected cells. A similar staining pattern is observed in cells expressing EGFP-cytochrome c.

results were promising, (as shown in Fig 5), we have not seen consistent cell killing by these constructs in cultured cells (and thus were reluctant to embark on costly animal studies). Hence, we are taking slightly different approaches that are detailed below:

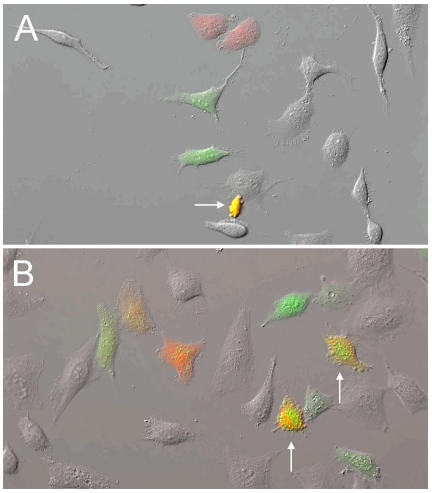


**Figure 5.** Apoptosis of cells co-expressing cytochrome c-EGFP and mCherry-heme lyase. HeLa cells were transfected with constructs encoding cytochrome c-EGFP and mCherry-heme lyase. Cells expressing either the cytochrome c (green) or heme lyase (red) appear healthy, while cells expressing both constructs (yellow; white arrows) demonstrate apoptotic morphology with membrane blebbing.

1) The overall focus of this grant was to exploit the cytochrome c sensitivity of breast cancer cells by either delivering cytochrome c genetically or identifying a small molecule that could mimic cytochrome c. We performed extensive cross-linking studies between Apaf-1 and cytochrome c as well as mutational studies of cytochrome c in an attempt to delineate the minimal determinants of binding. After several years of work, we believe that cytochrome c makes many contacts with Apaf-1 and that these multiple contacts may all be necessary for apoptosome nucleation. This may not bode well for identifying a small molecule mimetic. Hence, as we continue to screen for small molecules, we have begun working on the idea of delivering cytochrome c itself via liposomes to breast cancer cells. We have managed to package the cytochrome c in liposomes and will undertake assays to see if the protein can be delivered to breast cancer cells and, if so, if this will result in their death. To aid in these studies we have initiated a study with the laboratory of Liz Ledgerwood in New Zealand; they have discovered a natural variant of cytochrome c that is considerably more potent that wild type cytochrome c in triggering apoptosome activation. Delivery of this gene (or the protein) may offer more consistent and potent activation of apoptosis in breast cancer cells than wild type cytochrome c. Towards this end, a graduate student from Dr. Ledgerwood's laboratory will spend the month of September in my laboratory to characterize this protein in our various assays.

2) Because the goal of our proposal was to mimic or modulate apoptosome function for therapeutic benefit in breast cancer treatment, and the cytochrome c-based therapeutic approach has proven somewhat challenging, we undertook to find another way to activate caspase 9 specifically in cancer cells. For this purpose, we plan to engineer an altered Apaf-1-like protein that responds, not to cytochrome c, but to the activated HER2 receptor kinase, that is up-regulated/activated in many breast cancers. Expression of such a protein in breast tumors would be expected to eliminate tumor cells without affecting normal cells. The construct we will engineer is based on the finding that intiator caspases are activated through dimerization (under physiological conditions this dimerization is achieved following cytochrome c-mediated Apaf-1 oligomerization and recruitment of caspase 9 to the Apaf-1 CARD domain). Instead, as depicted in Fig 6, as a prototype, we have engineered a construct wherein tandem copies of the CARD domain of Apaf-1 are separated by a linker region that contains both a tyrosine phosphorylation site and a cognate SH2 domain. In the presence of high tyrosine kinase activity, the linker between the two Apaf-1 CARD domains is phosphorylated, and the SH2 binds intramolecularly to the tyrosine phosphate, juxtaposing the CARD domains, thereby dimerizing recruited caspase 9. We have engineered a family of these responsive to different

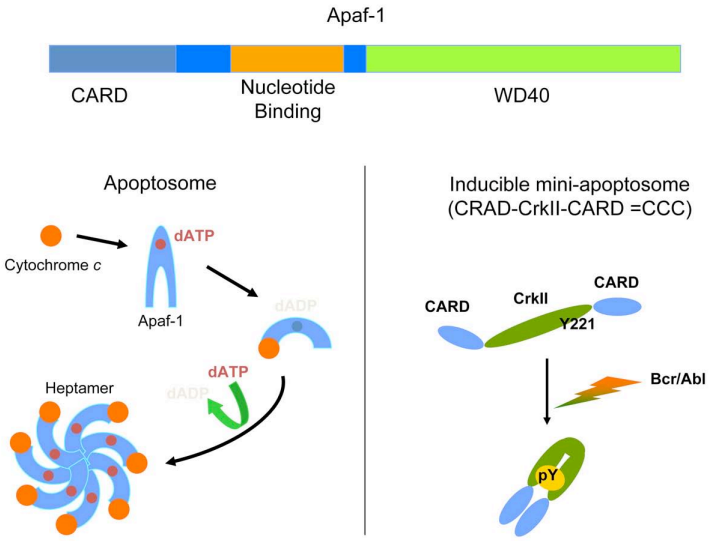


**Figure 6.** (Top) A schematic diagram of Apaf-1 protein is shown. Apaf-1 consists of the caspase-recruiting domain (CARD) at the N-terminal end, the nucleotide (dATP/dADP) binding domain at the middle section, and repeats of the WD40 domain at the C-terminal end. (Left) In cells, cytochrome c released from mitochondria binds to a monomeric Apaf-1 protein, which induces dATP hydrolysis and conformational changes of Apaf-1. Subsequently, a new dATP is loaded to Apaf-1 in place of dADP by a nucleotide exchange factor, triggering formation of Apaf-1 heptamers. Apaf-1 CARD recruits procaspase-9 into the heptameric Apaf-1 complex, resulting in oligomerization and activation of caspase-9. Activated caspase-9 can then cleave and activate executioner caspases to promote dismantling of the dying cell. (Right) An inducible mini-apoptpsome is depicted. This construct, named "CCC", consists of the adaptor protein CrkII flanked on both sides by Apaf-1 CARD. In this caspase variant, phosphorylation of Y221 on CrkII by Bcr-Abl is expected to cause intramolecular binding of pY221 to the Crk SH2 domain, producing a conformational change that juxtaposes the two CARD moieties. This intramolecular dimerization of CARD is then expected to recruit and activate pro-caspase-9 via induced proximity, resulting in the initiation of the caspase activation caspase to promote cell death.

tyrosine kinases (the specificity can be altered by changing the sequences around the tyrosine phosphorylation site and the relevant SH2 domain). As shown in Fig 6, our first iteration (in conjunction with a project unrelated to this grant) uses the tyrosine phosphorylation site and SH2 domains from the Crk adaptor protein. As shown in Fig. 7, this quite effectively kills cells overexpressing the Bcr-Abl tyrosine kinase that is active in leukemia's without damaging normal cells. We are now altering these sequences so that the tyrosine phosphorylation site is patterned on a HER2 autophosphorylation site and a cognate SH2 domain is built in. We anticipate that this will be highly effective in killing breast cancer cells overexpressing the HER2 receptor without damaging normal tissues.

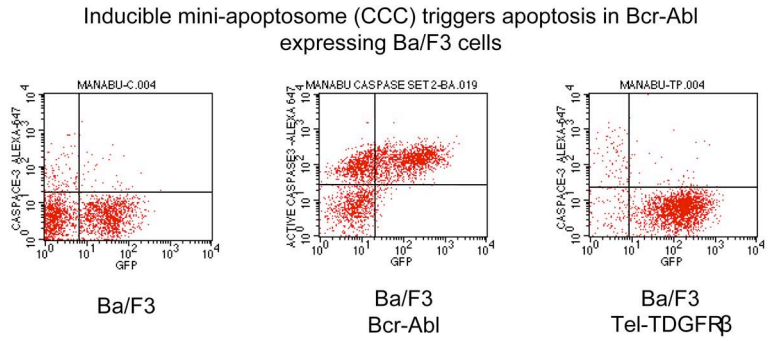


**Figure 7.** CCC specifically kills Ba/F3 cells expressing Bcr-Abl. Normal Ba/F3 cells or cells expressing Bcr-Abl or Tel-PDGFRbeta were infected with retroviral vector (MSCV-IRES-GFP) encoding CCC. Forty-eight hours after infection, the cells were fixed, stained with ant-active caspase-3 antibody, and analyzed by FACS

Key research accomplishments:

- Engineered heme lyase and cytochrome c-expressing constructs
- Tested constructs and observed some cell killing, but probably not enough for therapeutic utility
- Developed novel screening method for regulators of apoptosis
- Began engineering novel viral constructs for use as breast cancer therapeutic

Reportable outcomes:

Will be applying for follow-on funding from NIH